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Isolation and Activation of Fresh Fruit Bunch Oil Palm Acetyl Co-A Carboxylase

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ABSTRACT

Palm oil has a substantial contribution to national economy, as well as local economy development. At present the productivity of Indonesian oil plam plantation, especially small holderd is relatively low. Efforts to increase productivity can be done through biochemicals approach in this case enzymes that responsible for oil production. One of the bottle neck for oil production is the step converting acetyl co-A to malonyl co-A by acetyl co-A carboxylase (ACCs). ACCs in human is known to be activated with certain amino acid and organic acids, and divalen metals. For this reason this study was conducted to separate and study the activation of ACCs by those substances. Activity of ACCs was measured developed using HPLC method. Oil palm ACCs had 50 kDa of Biotin Carboxylase (BC) subunit dan 31 kDa of Carboxyl Transferase (CT) subunit. Separation of crude extract ACCs using dialysis and sephadex G-25 column chromatography showed the higher activity of enzyme. Addition of activator in the form of citric acid, Mg^{2+} and glutamic acid could stimulate activity of the enzyme.

***Keywords**: ACCs, separation, amino acids, tricarboxylic acids.*

1. INTRODUCTION

Indonesia is the largest palm oil producer in the world with annual production 33 million metric tonnes and global market share of 54 % in 2015/2016 [1]. Indonesia export volume in 2014 was 22,9 million tonnes with the value 17,4 million US$ or 75% of total Indonesian oil palm production [2]. Oil palm has a substantial national economy contribution, as well as local economy development [3]. The important of social role of oil palm contribution can be seen from the small holder plantation area that reached 39% from total plantation in Indonesia. Among the other plantations, small holders productivity was the lowest, on average only 3,14 ton/ha meanwhile private productivity was 3.9 ton/ha and state owned 3.89 ton/ha [1].

The efforts to increase productivity can be done through biochemicals approach in this case enzymes that responsible for oil production. One of the bottle neck for oil production is convertion acetyl co-A to malonyl co-A by Acetyl co-A carboxylase (ACCs). This step is the rate commited step reaction [4]. ACCs [EC 6.4.1.2] is found in all the kingdoms of life excepting the archaea (which have isoprenoid lipids in place of lipids based on fatty acids). In plant, ACCs is required for the synthesis of a variety of compounds other than fatty acids, for example terpenoid, flavonoid, etc. The first ACCs studied were those of mammals in which the overall reaction is catalyzed by a single very large protein [5]. The ACC reaction consists of two discrete half-reactions. In the first half-reaction, biotin is carboxylated by bicarbonate in an ATP dependent reaction to form carboxybiotin whereas in the second half reaction, the carboxyl group is transferred from carboxybiotin to acetyl Co-A to form malonyl Co-A [6]. Some researchers had shown that over-expression of acetyl CoA carboxylase can increase fatty acid production and TAG increase by 5 times in transgenic potatoe [7]. Nakaew et al. (2008) concluded that expression of encoded subunit, biotin carboxylase (AccD), part of ACCs that catalyze synthesis of fatty acids and can be used as marker in oil palm breeding [8]. Meanwhile, Ruenwai et al. (2009) concluded that there was a 40% increase in the total fatty acids in the Hansenula yeast polymorpha after the ACCs gene was transformed from Mucor rouxii [9]. Therefore, increasing the activity of the ACCs enzyme is considered to be able to significantly increase the productivity of oil-producing plants, especially oil palm.

Most of ACCs studies were on diabetic and diets in mammals enzyme [10]. In animals, two isoforms of the carboxylase have been identified, ACC1 (Mr

265,000) and ACC2 (Mr 280,000) [11]. The ACC1 carboxylases are highly expressed in lipogenic tissues, such as liver and adipose, and their levels are regulated transcriptionally while their activities are regulated posttranslationally by dephosphorylation of selected serine residues and by allosteric regulation through the action of citrate and palmitoyl-CoA. Dietary and hormonal states of the animal affect the level and activities of the ACC1 enzymes. A carbohydrate-rich, low-fat diet stimulates the expression and activities of ACC1, whereas starvation and diabetes reduce the ACC1 activities by repressing the expression of the ACC1 gene or by increasing the phosphorylation levels of the ACC1 protein (or both). Treating diabetic animals with insulin increases the activity of the enzyme either by dephosphorylation of the protein or by activation of it with citrate (or both), and prolonged insulin treatment stimulates the synthesis of ACC1 protein.

Kowluru et al. (2001) reported the addition of certain amino acids and divalent metals, activated beta cell ACCs in human pancreas [12]. The same results were also obtained by Vavvas et al. (1997) where divalent metals and amino acid PP2A enzymes regulate ACCs activation in muscles [13]. Tests conducted by Santoso et al. (2018) shows that the addition of these activators increases oil production on oil palm by 45% [14]. For this reason, this study will study how activation of amino acids and certain organic acids, as well as divalent metal on the activity of oil palm fruit ACCs enzymes.

2. MATERIALS AND METHODS

2.1 Materials

Fresh fruit bunches of oil palm harvested from the Ciomas experimatation of IRIBB in Bogor, Acetonitrile, KH_2PO_4, methanol, N_2, Tris HCl pH 8.3, 10% [v/v] glycerol,mercaptoethanol, Na_2EDTA, acetyl Co-A, malonyl Co-A, 50 µM Tris pH 7, ATP, $KHCO_3$, $MgCl_2$, dithiotreitol, bovin serum albumin, perchloric acid, glutamic acid, citric acid, succinic acid, $MgSO_4$, $CaCl_2$ at p.a grade purchased from local supplier.

2.2 Methods

2.2.1 Optimization of acetyl Co-A dan malonyl Co-A using HPLC.

Optimization of HPLC conditions was carried out in determining the optimal flow rate, eluent (acetonitrile, KH_2PO_4, and methanol) and isocratic or solvent gradients so that a good separation of acetyl CoA and malonyl CoA was obtained[15].

2.2.2 Isolation and partial purification of the ACCs enzyme.

Palm fruit mesocarp (aged 15-17 weeks WAA) was cut into small pieces and then mashed using a coffee smoothing tool (Philips HR 2115 G). The mesocarp powder was transferred to the centrifuge tube and homogenized with buffer (100 mM Tris HCl-KOH, pH 8.3, 10% [v / v] glycerol, 10 mM, B-mercaptoethanol, 1 mm Na_2EDTA), then centrifuged at 20,000 g and 2 ºC for 30 minutes. The extracted supernatant is transferred into the eppendorf tube and stored in the freezer until used. The protein concentration was determined by Bradford method and characterized by SDS PAGE [16].

2.2.3 Measurement of ACCs activity from palm fruit mesocarp.

ACCs activity from extracts of palm fruit mesocarp protein was determined using HPLC by measuring the speed of the decrease in acetyl Co-A or the increase in malonil Co-A concentration. The protein extract was reincubated at 25ºC for 30 minutes with 2 mg/L bovine serum albumin and 10 mM potassium citrate. The reaction was started by transferring 50 µL of extract incubated into the reaction mixture (50 µM Tris, pH 7.5. 6 µM acetyl co-A, 2 mM ATP, 7 mM $KHCO_3$, 8 mM $MgCl_2$, 1 mM dithiotreitol and 1 mg/mL of serum albumin bovine) up to a total volume of 200 µL, then incubated for 5-20 minutes at 25ºC. The reaction was stopped by adding 50 µL of 10% perchloric acid, then centrifuged at 10,000 g for 3 minutes. The supernatants are used for HPLC analysis [15].

2.2.4 Effect of adding activators to ACCs activity.

Activators to be added are divalent metals (Ca^{2+} and Mg^{2+}), amino acids (glutamate), and organic acids (citric and succinic acid) with variations in concentrations.

3. RESULTS AND DISCUSSION

3.1 Optimization of HPLC for cetyl co-A and malonyl co-A analysis

The ACCs activity testing technique that is widely developed today through the use of radioactive [14C] bicarbonate that is used as a substrate for extending carbon chains from acetyl to malonyl co-A (Davis et al., 2000). Levert et al. (2002) developed a method of measuring activity using HPLC. To produce a good separation, optimization was carried out to separate the acetyl coA peaks as substrate from malonyl CoA as products. Acetyl Co-A is relatively more polar than malonyl CoA because of the smaller amount of

carbon. Initial tests using the Levert et al., 2002 method, acetyl CoA was not separated well from malonyl CoA in the corresponding bands. For this reason, gradient solvent testing (Figure 1) was carried out, but still the resolution was not good enough to meet resolution requirements (<1.5) [15]. Then isocratic eluents produced the best separation were KH_2PO_4: acetonitrile = 90: 10 v / v (Figure 2A) and repetition still showed consistent results (good repeatability) (Figure 2). The standard curves of acetyl and malonyl co-A could used the quantification standard with R values of 0.995251 and 0.999722, respectively (Figure 3).

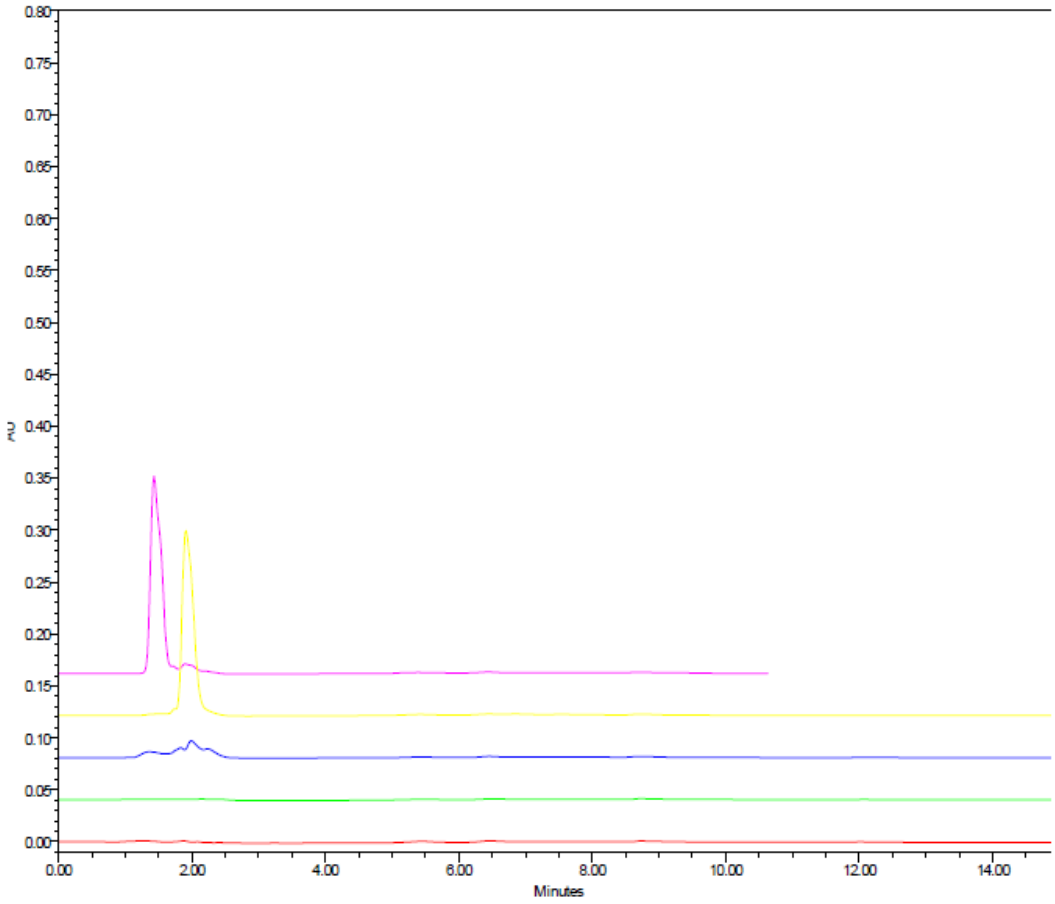

Figure 1. Separation acetyl coA with malonyl coA using aquadest, KH2PO4, methanol and acetonitrile solvents, standard acetyl coA (yellow) and malonyl CoA (pink), λ 230 nm, v: 1 ml / minute.

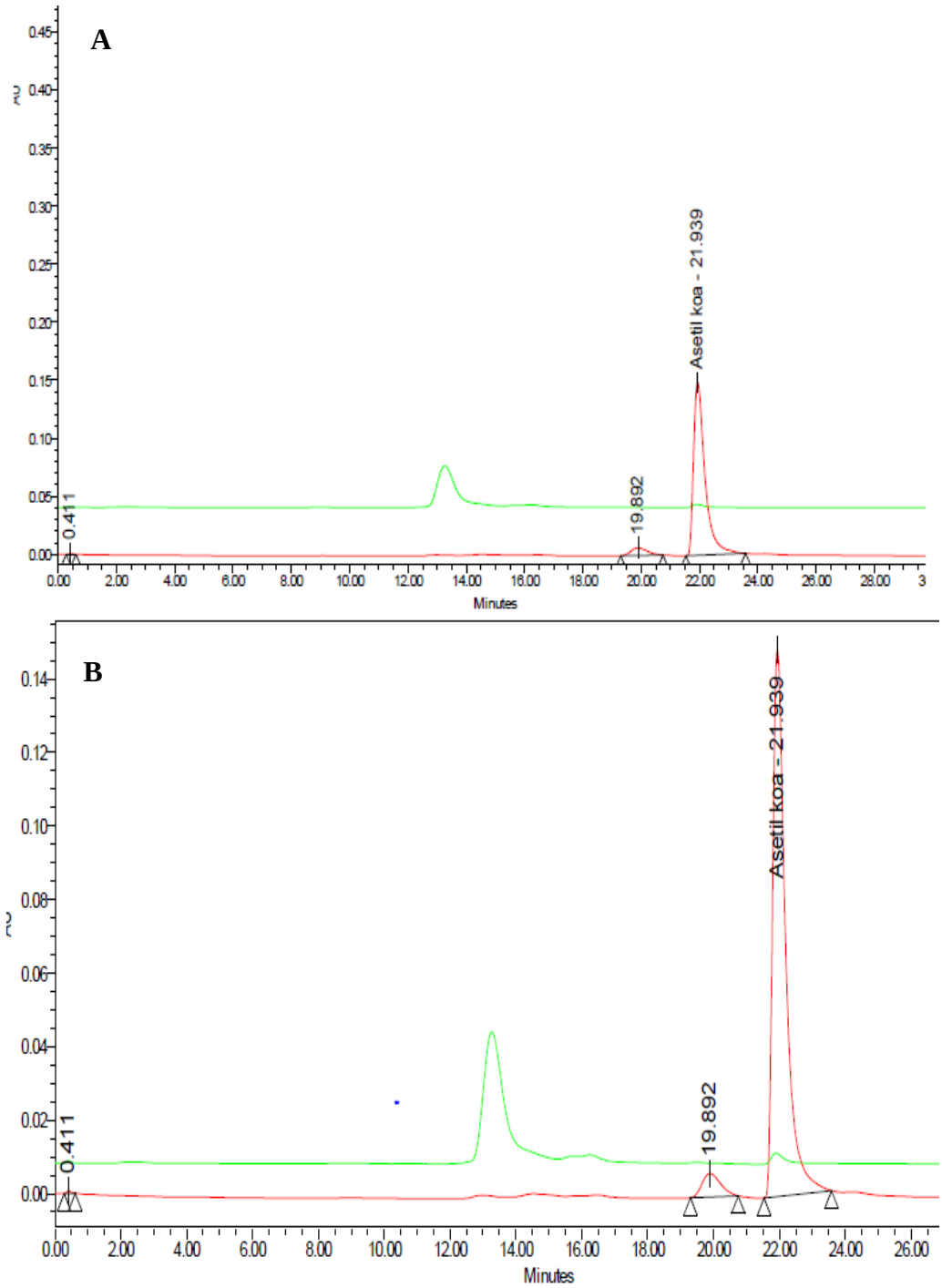

Figure 2. Separation acetyl coA with malonyl coA using gradient of KH2PO4 and acetonitrile = 90:10 v / v, standard acetyl co-A (green) and malonyl co-A (pink), λ 230 nm, v: 1 ml / minute.

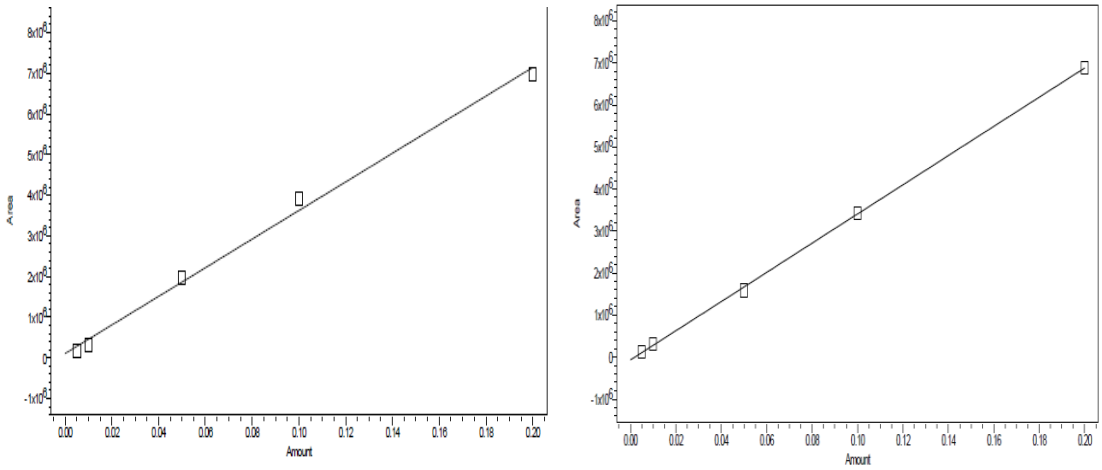

Figure 3. Standard curves of acetyl and malonyl coA with each regression Y = 3.52e + 007 X + 1.03e + 005 (R ^ 2: 0.995251) and Y = 3.47e + 007 X - 6.71e + 004 (R ^ 2 : 0.999722).

3.2 Isolation and partial purification of the ACCs enzyme

The protein profile in mature and young fruit was showed differences in the protein profile (Figures 4 A and B). Protein expression did not increase with increasing fruit maturity, a similar finding was reported by Budiani et al., (2004) where the total protein concentration did not increase with increasing oil content and concluded during the fruit development stage [16], some of the proteins involved in oil synthesis increased in expression. while other proteins decreased. ACCs consists of 4 sub-units (BC, CT, CPP). BC is known to have a size of 50 kDa, meanwhile CT reported have a size130 kDa and consists of molecules 30 and 35 kDa in the form of a2b2. The size of this sub-unit is different from the size of the BC liver of mice which reached 215 kDa which is a dimer of several sub-units 118-125 kDa [17].

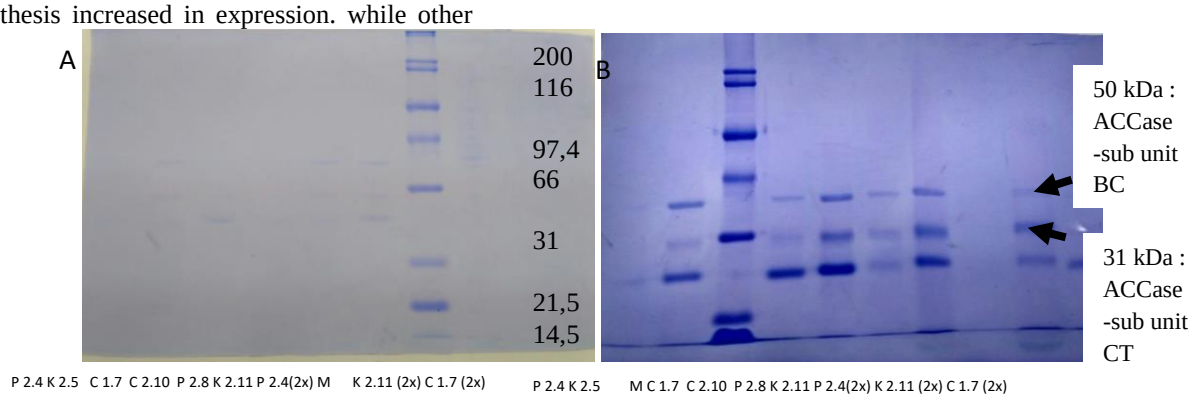

Figure 4. SDS PAGE profile of ripe fruit (A) and young fruit (B).

The protein produced was partially purified using precipitation withNH_4SO_4 and dialysis. SDS damages the secondary, tertiary and quaternary structure of the protein to produce a negatively charged linear polypeptide chain that is enveloped in the SDS molecule, and also mercapto ethanol that contributes to denaturing proteins by reducing disulfide bonds. For this reason, Native PAGE was used to maintain the secondary structure of protein and charge density of "native" protein (Figure 5). After precipitation, crude protein was dialyzed and fractionated through sephadex chromatography coloumn to separate the small molecules (acetyl and malonil co-A, avoiding interference in activity measurement process (Figure 6). Based on data from Figure 6, fractions 7 and 8 were fractions with the highest concentration values. While the fraction with the lowest protein concentration is fraction 1.

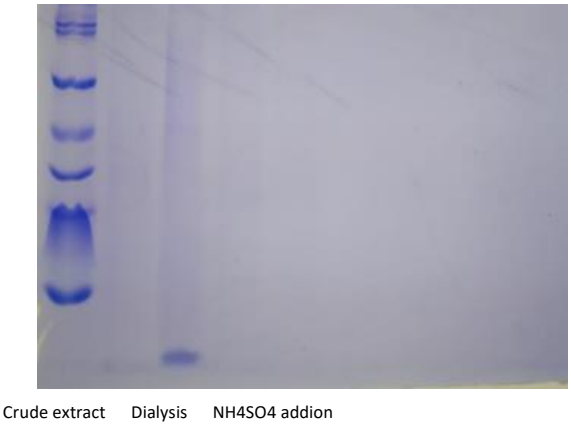

Figure 5. Profile of Native PAGE resulting from the separation of crude extract, crude extract of dialysis results and addition of NH_4SO_4.

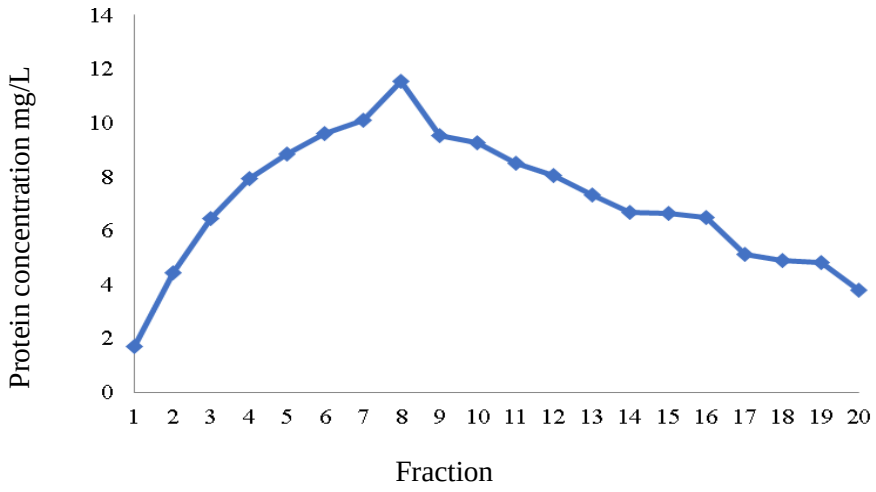

Figure 6. The sephadex G-25 column chromatography protein concentration curve.

3.3 Measurement of ACCs activity from palm fruit mesocarp treated with activators

Previous study on the addition of previous activators on animal ACCs enzymes and it was concluded that the addition of citric acid can increase ACCs activity because citrate allows dephosphorylation. Phosphorylation causes a decrease in Vmax and increases $K_{0.5}$ [18]. The activity of palm fruit ACCs also increase with the addition of citrate, in contrast to organic acids such as succinate (Table 1). Citrate was assumed stimulate the reaction of both the enzyme partial reaction and this compound stabilizes the active conformation of the carboxylated enzyme. Free citrate can also form complexes with $Mg^{2+,}$ where Mg-ATP is one of the substrate of ACCs enzyme activity [19]. In addition to citric acid the addition of divalent metal Mg^{2+} increases ACCs activity compared to the addition of Ca^{2+} metal (Table 2). The effect of Mg is greater than Ca because Mg is a cofactor of biotin carboxylase.

Table 1. Effect of the addition of citric acid and suucinic acid to ACCs activity.

Organic acids	Enzyme activity unit/mL
Citric acid	
1 µL	0.1189
5 µL	0.0006
10 µL	0.0048
Succinic acid	
1 µL	000001
5 µL	-0.00001
10 µL	-0.00002

Table 2. Effect of addition of Mg and Ca metals to ACCs activity.

Divalen ion	Enzyme activity unit/mL
Mg	
10 µL	0.0023
20 µL	0.0106
30 µL	0.0133
Ca	
10 µL	0.0081
20 µL	0.0005
30 µL	0.0040

Meanwhile the addition of glutamic acid tends to increase ACCs activity (Table 3). According to Gaussin et al. (1996), glutamate activates protein phosphatase (GAPP) which removes phosphate groups and activates ACCs [20]. Kowluru et al. (2001) also reported that PP2 phosphatases that are sensitive to glutamate and magnesium dephosphorylate and activate ACC in pancreatic beta cells in humans.

Table 3. Effect of addition of glutamic acid to ACCs activity.

Glutamic acid	*Enzyme activity unit/mL*
2 µL	3.11E-05
5 µL	2.77E-03
10 µL	4.75E-03

The higher the glutamate concentration, the higher the ACCs activity. The separation process showed that fraction 7 and 8 had higher ACCs activity than crude extract and dialysis results (Table 4) and the fractionation results showed a significant increase in the addition of glutamate (Figure 4.8).

Table 4. ACCs activity in the stages of separation of the ACCs enzyme from palm fruit.

	Enzyme activity U/mL
Crude extract	9.41E-06
Dialysis	2.88E-05
Fraction 7	3.27E-05
Fraction 8	4.12E-05

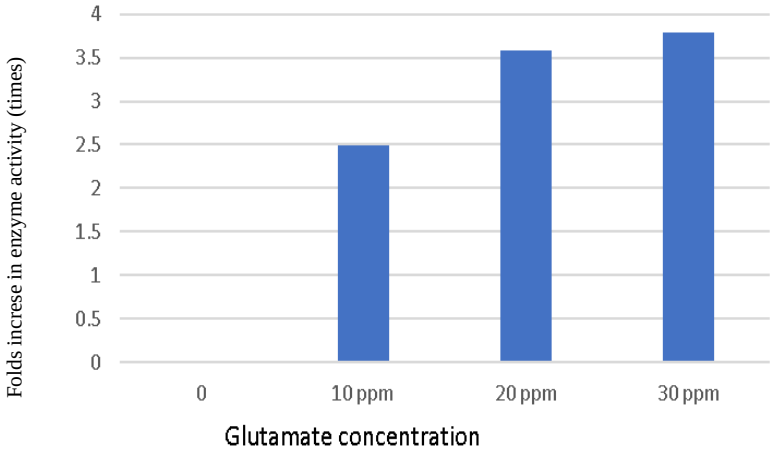

Figure 7. ACCs activity of fraction resulting from separation with the addition of glutamic acid.

4.CONCLUSSIONS

Activity of ACCs measurement was developed using HPLC metho with non-radioactive substrate. Oil palm ACCs had 50 kDa of BC subunit dan 31 kDa of CT subunit. Separation of ACCs from crude extract using dialysis and sephadex G-25 column chromatography showed the higher activity of enzyme. Addition of activator in the form of citric acid, Mg^{2+} and glutamic acid can stimulate enzyme activity.

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